

Comparison of Genospecies and Antimicrobial Resistance Profiles of Isolates in the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* Complex from Various Clinical Specimens

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This study was conducted to compare the prevalences of antimicrobial resistance profiles of clinical isolates in the *Acinetobacter calcoaceticus*-*Acinetobacter baumannii* complex from sterile and nonsterile sites and to further study the relationship of antimicrobial resistance profiles and genospecies by amplified rRNA gene restriction analysis (ARDRA). A total of 1,381 isolates were tested with 12 different antibiotics to show their antimicrobial susceptibility profiles. A total of 205 clinical isolates were further analyzed by ARDRA of the intergenic spacer (ITS) region of the 16S-23S rRNA gene. It was found that the overall percentage of isolates from nonsterile sites (urine, sputum, pus, or catheter tip) that were resistant to the 12 antibiotics tested was significantly higher than that of isolates from sterile sites (cerebrospinal fluid [CSF], ascites fluid, and bloodstream) (46% versus 22%; $P < 0.05$). After ARDRA, it was found that 97% of the 62 isolates resistant to all antibiotics tested were the *A. baumannii* genospecies, which was identified in only 31% of the isolates susceptible to all antibiotics tested. More genospecies diversity was identified in the isolates susceptible to all antibiotics tested, including genospecies of 13TU (34%), genotype 3 (29%), and *A. calcoaceticus* (5%). Furthermore, as 91% (10/11) of the isolates from CSF were susceptible to all antibiotics tested, the *A. calcoaceticus*-*A. baumannii* complex isolates with multidrug resistance could be less invasive than the more susceptible isolates. This study also indicated current emergence of carbapenem-, fluoroquinolone-, aminoglycoside-, and cephalosporin-resistant *A. calcoaceticus*-*A. baumannii* complex isolates in Taiwan.

Members of the genus *Acinetobacter* have been recognized to be widespread and persistent on a variety of surfaces and inanimate environments (4, 42). The bacteria have recently become one of the emerging nosocomial pathogens (1, 28). Higher mortality could be observed in patients with a long period of hospitalization, on mechanical ventilation, on chemotherapy, and with underlying diseases (8, 29, 33).

Acinetobacter spp. are glucose-nonfermentative, nonfastidious, catalase-positive, oxidase-negative, strictly aerobic Gram-negative coccobacilli and commonly occur in diploid formation or in chains of variable length (32). However, different genospecies cannot be easily identified using traditional methods. Members of the genus have been classified in various ways, and therefore it is difficult to understand the true status of the epidemiology and clinical importance of these organisms (41). Since 1986, the taxonomy of the genus *Acinetobacter* has undergone extensive revision (2). The original single species named *Acinetobacter calcoaceticus* has been abandoned, and at least 32 genospecies have now been proposed, 17 of which have been correlated with species names. Identification of the members of the genus *Acinetobacter* to the species level by traditional methods is problematic (5, 10). Among the different genospecies, *A. baumannii* and its close relatives (genospecies 3 and 13TU), together forming the “*A. calcoaceticus*-*A. baumannii* complex,” belong to the molecular group of greatest clinical importance, which accounts for the vast majority of human infections and hospital outbreaks (13, 31, 39).

In recent years, clinical cases due to *A. calcoaceticus*-*A. baumannii* complex infection have raised major concerns worldwide.

The infection can be fatal, and clinical *A. calcoaceticus*-*A. baumannii* complex isolates were frequently resistant to the last-line antibiotics for clinical treatment, including cephalosporins, aminoglycosides, fluoroquinolones, and carbapenems (30). In Taiwan, several studies further indicated the emergence of the infection, especially with regard to multidrug resistance and nosocomial infections (14, 15, 17). Since 1998, several studies on isolates in the *A. calcoaceticus*-*A. baumannii* complex have been conducted in Taiwan, and several genospecies associated with antibiotic resistance have been identified (11, 24, 26). The genotyping methods included restriction analysis of the amplified rRNA gene (amplified rRNA gene restriction analysis [ARDRA]) (3, 40), the 16S-23S rRNA gene intergenic spacer (ITS) region (6), and the whole ribosomal operon (9). ARDRA is easy to conduct and can be considered a useful tool for clinical identification. Through molecular epidemiology, trends in the prevalence of antibiotic resistance in different genospecies have also been documented (24, 26).

Clinically, it is important to differentiate the different genospecies of isolates in the *A. calcoaceticus*-*A. baumannii* complex. The

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TABLE 1 Comparison of percentages of antibiotic-susceptible *A. calcoaceticus*-*A. baumannii* complex isolates from different specimens

Susceptibility to all 12 antibiotics tested ^a	No. (%) of isolates from:											
	Nonsterile areas						Sterile areas					
	Bile	Tip	Urine	Pus	Sputum	Total	Body Fluid	Ascites	Pleural fluid	CSF	Bloodstream	Total
Susceptible	0 (0)	18 (11)	15 (7)	24 (11)	13 (3)	70 (7)	7 (58)	4 (22)	3 (16)	11 (44)	116 (36)	141 (35)
Resistant	4 (36)	78 (46)	68 (32)	103 (47)	204 (55)	457 (46)	2 (17)	6 (33)	5 (26)	6 (24)	69 (21)	88 (22)
Total	11	170	210	219	373	983	12	18	19	25	324	398

^a The antibiotics tested were amikacin, ceftazidime, ciprofloxacin, colistin, cefepime, gentamicin, imipenem, levofloxacin, meropenem, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, and piperacillin-tazobactam.

main reason is that different genospecies have different biological characteristics and pathogenicity, which could be relevant to treatment efficacy (11). The previous study indicated that genospecies 3 and 13TU had different antibiotic susceptibilities (2, 14). Therefore, there is an urgent need to conduct molecular epidemiological investigations to understand the relationship of genospecies and their antimicrobial resistance profiles. The results will be helpful for better clinical treatment and control of the infection (7, 11, 18).

The objectives of this study were to compare the prevalences of antimicrobial resistance profiles of isolates in the *A. calcoaceticus*-*A. baumannii* complex from sterile and nonsterile sites in patients and to further study the relationship of antimicrobial resistance profiles and genospecies by amplified rRNA gene restriction analysis (ARDRA) of the intergenic spacer (ITS) region of the 16S-23S rRNA gene.

MATERIALS AND METHODS

Collection of isolates in the *A. calcoaceticus*-*A. baumannii* complex and antibiotic susceptibility testing. A total of 1,381 isolates in the *A. calcoaceticus*-*A. baumannii* complex from clinical specimens from various sites in patients were collected at Teaching Hospital of China Medical University, Taiwan, in 2008. The hospital is a medical center in central Taiwan with more than 2,000 beds. The BD Phoenix NMIC/ID-2 commercial kit (Becton, Dickinson Diagnostic Systems, Sparks, MD) was used for antibiotic susceptibility testing. The antibiotics tested included amikacin (AN) (MIC, 8 to 32 µg/ml), ceftazidime (CAZ) (0.5 to 16 µg/ml), ciprofloxacin (CIP) (0.5 to 2 µg/ml), colistin (CL) (1 to 4 µg/ml), cefepime (FEP) (2 to 16 µg/ml), gentamicin (GM) (2 to 8 µg/ml), imipenem (IPM) (1 to 8 µg/ml), levofloxacin (LVX) (0.5 to 2 µg/ml), meropenem (MEM) (1 to 8 µg/ml), ampicillin-sulbactam (SAM) (4/2 to 16/8 µg/ml), trimethoprim-sulfamethoxazole (SXT) (0.95/9.5 to 2/38 µg/ml), and piperacillin-tazobactam (TZP) (4/4 to 64/4 µg/ml).

DNA extraction and ARDRA for the ITS region of the 16S-23S rRNA gene of isolates in the *A. calcoaceticus*-*A. baumannii* complex. A total of 205 clinical isolates (8 from ascites fluid, 11 from cerebrospinal fluid [CSF], and 186 from bloodstream) collected from 2007 to 2009 were further analyzed by amplified rRNA gene restriction analysis (ARDRA) of the intergenic spacer (ITS) region of the 16S-23S rRNA gene (6). All the isolates and reference strains used in this study were cultured and identified to be in the *A. calcoaceticus*-*A. baumannii* complex according to a previous study (32). Briefly, isolates were grown on BAP agar plates (Becton, Dickinson and Company, Sparks, MD), and then pure bacterial colonies were suspended in 600 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and the pellet was obtained after brief centrifugation using a microcentrifuge. DNA was extracted from the pellet with a Genomic DNA Mini Kit (Geneaid, New Taipei City, Taiwan) following the manufacturer's recommendations. The DNA extracts were stored at -20°C for further molecular analysis. For genospecies identification of the isolates, ARDRA for the ITS region of the 16S-23S rRNA gene was applied as

previously described by Vaneechoutte et al. (40). Briefly, the specific segment was amplified with primer set 5'-TGGCTCAGATTGAACGCTGG CGGC-3' and 5'-TACCTTGTTACGACTTCAACCCCA-3'. Amplification was performed using 5 µl of DNA extract in a 50-µl PCR mixture containing 1 U of *Taq* polymerase (Fermentas, Vilnius, Lithuania), 200 mM each deoxynucleoside triphosphate (Viogen, New Taipei City, Taiwan), and 0.2 mM each primer in reaction buffer (1.5 mM MgCl₂ and 50 mM KCl in 10 mM Tris-HCl, pH 8.3). The PCR conditions were established as follows: first denaturation at 94°C for 5 min, 35 cycles of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, and extension at 72°C for 1 min, and final extension at 72°C for 7 min. The expected amplified product was approximately 1,500 bp. Three restriction endonucleases, *AluI*, *HhaI*, and *MboI* (New England BioLabs, Beverly, MA), were used for DNA digestion following the manufacturer's instructions. Using 5 µl of a digested PCR product, the restriction fragment pattern of each sample was visualized by 2% agarose (Amresco Inc., Solon, OH) gel electrophoresis at 100 V for 30 min in Tris-borate-EDTA (TBE) buffer. Genospecies were determined by the digestion patterns as described by Vaneechoutte et al. (40).

RESULTS

Analysis of antibiotic susceptibility results for isolates from different clinical specimens. After using antibiotic susceptibility testing to analyze a total of 1,381 isolates from 2008, the percentages of resistance were found to be 68% for amikacin, 75% for ceftazidime, 73% for ciprofloxacin, 0% for colistin, 75% for cefepime, 75% for gentamicin, 65% for imipenem, 71% for levofloxacin, 68% for meropenem, 67% for ampicillin-sulbactam, 74% for trimethoprim-sulfamethoxazole, and 78% for piperacillin-tazobactam. As shown in Table 1, among the 983 isolates from nonsterile sites, the highest percentage of resistance to all antibiotics tested was in sputum, followed by pus and central venous pressure (CVP) catheter tips. The highest percentage of resistance of all antibiotics tested for the 398 isolates from sterile sites was in ascites fluid, followed by pleural fluid and cerebrospinal fluid (CSF). The overall percentage of isolates from nonsterile sites that were resistant to all antibiotics was significantly higher than that of isolates from sterile sites (46% versus 22%; $P < 0.05$).

Molecular typing of isolates by ARDRA. After analyzing 205 isolates (including 62 isolates resistant to the 12 antibiotics tested and 143 isolates susceptible to the 12 antibiotics tested) for genospecies identification by ARDRA, except for one isolate with an undetermined genospecies, 97% of the all resistant isolates were the unique *A. baumannii* genospecies (Table 2). However, isolates susceptible to all antibiotics tested had more genetic diversity; the major genospecies determined by ARDRA was 13TU (34%), followed by *A. baumannii* (31%), genotype 3 (29%), and *A. calcoaceticus* (5%). 13TU, *A. baumannii*, genotype 3, and *A. calcoaceticus* could all be identified in blood isolates. However, isolates from ascites fluid or CSF were only *A. baumannii* and genotype 3.

TABLE 2 Comparison of genospecies of *A. calcoaceticus*-*A. baumannii* complex isolates in resistant and susceptible groups from different clinical sources

Susceptibility to all 12 antibiotics tested ^a	Source of specimens	Genospecies (no. of isolates)
Resistant (<i>n</i> = 62)	CSF	Undetermined (1)
	Bloodstream	<i>A. baumannii</i> (56), untypeable (1)
	Ascites fluid	<i>A. baumannii</i> (4)
Susceptible (<i>n</i> = 143)	CSF	<i>A. baumannii</i> (5), genotype 3 (5)
	Bloodstream	13TU (49), <i>A. baumannii</i> (37), genotype 3 (35), <i>A. calcoaceticus</i> (7), undetermined (1)
	Ascites fluid	<i>A. baumannii</i> (2), genotype 3 (2)

^a The antibiotics tested were amikacin, ceftazidime, ciprofloxacin, colistin, cefepime, gentamicin, imipenem, levofloxacin, meropenem, ampicillin-sulbactam, trimethoprim-sulfamethoxazole, and piperacillin-tazobactam.

Further analysis of the results by year (Table 3) showed that the *A. baumannii* genospecies with resistance to all 12 antibiotics tested was dominant in 2007 and 2008. However, in 2009, isolates were still mainly of the *A. baumannii* genospecies but were susceptible to all 12 antibiotics tested.

DISCUSSION

In Taiwan, the emergence of infections due to *Acinetobacter* bacteria has recently been emphasized with respect to the aspects of multidrug resistance and nosocomial infections (12, 14, 15, 20). Nevertheless, most clinical microbiological laboratories using traditional methods or commercial identification systems could not easily distinguish various genospecies of the isolates in the *A. calcoaceticus*-*A. baumannii* complex (32). Therefore, further genotyping of isolates in the *A. calcoaceticus*-*A. baumannii* complex may offer more clinical information. This study first identified that the overall percentage of resistance to all antibiotics tested was significantly different between the isolates from sterile sites and nonsterile sites (sterile sites, 22%; nonsterile sites, 46% [*P* < 0.05]). Furthermore, it was found that isolates resistant to all antibiotics tested were mostly of the unique *A. baumannii* genospecies, but more genospecies could be identified in isolates susceptible to all antibiotics tested.

In this study, very high percentages of resistance to imipenem, meropenem, amikacin, and ceftazidime (65%, 68%, 68%, and 75%, respectively) were identified in isolates from 2008. It is important to note that these are the last-line agents for clinical treatments. Previous studies conducted in intensive care units (ICUs) of several teaching hospitals in Taiwan had already indicated that

the percentage of resistance to imipenem in *A. baumannii* was increasing, from 22% in 2000 to 25% in 2005 (16, 21). In other countries, such as Estonia, 5% of *A. baumannii* strains were resistant to meropenem and imipenem (27). Furthermore, in the MYSTIC report from 48 European hospitals for isolates from the period of 2002 to 2004 (38), 27% and 30% of isolates were resistant to meropenem and imipenem, respectively. Therefore, such high percentages of resistance in the current study, compared to the previous findings in Taiwan as well as in other countries (16, 21, 27, 38), may suggest that the prevalence of the isolates in the *A. calcoaceticus*-*A. baumannii* complex that are resistant to carbapenems has recently been increasing in Taiwan. In our hospital, the prevalence of isolates in the *A. calcoaceticus*-*A. baumannii* complex that were resistant to imipenem was below 25% before 2004 but abruptly increased to 57% in 2006 and approached 71% in 2011 (unpublished data). In addition, most of the isolates in the *A. calcoaceticus*-*A. baumannii* complex were resistant to fluoroquinolones (76%), aminoglycosides (75%), and cephalosporins (77%). The situation is very similar to what has been found in Europe, where 68%, 66%, and 52% of the isolates were resistant to ceftazidime, ciprofloxacin, and gentamicin (38).

A previous study (22) applied the oligonucleotide array system to characterize 52 blood isolates in the *A. calcoaceticus*-*A. baumannii* complex and found that antimicrobial susceptibility profiles were different among different genospecies of the *A. calcoaceticus*-*A. baumannii* complex. Nevertheless, limited information is currently available to further explore whether *A. calcoaceticus*-*A. baumannii* complex isolates from different collection sites can be associated with different antimicrobial resistance characteristics. In this study, it was found that the overall percentage of isolates from nonsterile sites that were resistant to all antibiotics was significantly higher than that of isolates from sterile sites (46% versus 22%; *P* < 0.05). However, the overall percentage of isolates from sterile sites that were susceptible to all antibiotics tested was significantly higher than that of isolates from nonsterile sites (35% versus 7%; *P* < 0.05). After ARDRA, the *Acinetobacter* isolates from sterile sites (CSF, bloodstream, and ascites fluid) showed genetic diversity. Furthermore, compared to all resistant strains, more genetic diversity was identified in all susceptible strains. The results may imply a close relationship of genospecies and antimicrobial resistance profiles. Several *Acinetobacter* ribotypes have been reported to be associated with pan resistance (35). Lim et al. (25) also reported that the majority of MBL-producing *Acinetobacter* isolates were *Acinetobacter* phenon 6/ct 13TU, genospecies, 3 and 13TU. The study also documented that 40 strains of *A. baumannii* showed resistance to most antimicrobial agents, except carbapenems, but isolates of other genospecies, such as *Acineto-*

TABLE 3 Genospecies distribution of *A. calcoaceticus*-*A. baumannii* complex isolates, 2007 to 2009

Genospecies	No. (%) of isolates resistant or susceptible ^a in:					
	2007 (<i>n</i> = 53)		2008 (<i>n</i> = 82)		2009 (<i>n</i> = 70)	
	Resistant	Susceptible	Resistant	Susceptible	Resistant	Susceptible
<i>A. baumannii</i>	22 (42)	5 (9)	30 (37)	9 (11)	9 (13)	38 (54)
<i>A. calcoaceticus</i>	0 (0)	0 (0)	0 (0)	7 (9)	0 (0)	0 (0)
Genotype 3	1 (0)	13 (25)	0 (0)	14 (17)	0 (0)	15 (21)
13TU	2 (0)	9 (17)	0 (0)	22 (27)	0 (0)	18 (26)
Undetermined	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

^a Isolates resistant or susceptible to the 12 antibiotics tested in this study.

bacter phenon 6/ct 13TU, genospecies 3, and 13TU, were usually susceptible to ciprofloxacin and ampicillin-sulbactam. Similarly, we also found that isolates resistant to all 12 antibiotics tested were mainly of the *A. baumannii* genospecies (97%), but only 31% of the isolates susceptible to all antimicrobials tested were *A. baumannii* genospecies.

In this study, 10 isolates from CSF were all susceptible to the 12 antimicrobials tested. This may raise the concern that the *A. calcoaceticus*-*A. baumannii* complex isolates that are susceptible to more antibiotics could be more invasive than the isolates with multidrug resistance. The phenomenon has never been discussed for isolates in the *A. calcoaceticus*-*A. baumannii* complex, but it has clinical importance. In human uropathogenic *Escherichia coli* (UPEC), chloramphenicol-, tetracycline-, and streptomycin-resistant isolates were associated with reduced virulence potential, and their virulence-related genes were found to be less frequent (37). Similarly, a study by Piatti et al. (34) also indicated that quinolone-resistant *E. coli* strains harbored fewer virulence genes than the susceptible strains. Future research should be conducted to further elucidate whether the same is the case for *Acinetobacter* bacteria.

Previous studies applied multilocus sequence typing (MLST) (43), randomly amplified polymorphic DNA (RAPD) (36), amplified fragment length polymorphism (AFLP) (19), and *rpoB* gene sequence (23) analysis for the identification of clinically important and emerging *A. calcoaceticus*-*A. baumannii* complex isolates. These methods are time-consuming and are not easy to apply in clinical settings. MLST analysis even needs to include analysis of sequences of several housekeeping genes (*gltA*, *gyrB*, *gdhB*, *recA*, *cpn60*, *gpi*, and *rpoD*). The ARDRA using 5 different restriction enzymes offers an efficient way to differentiate genospecies of the *A. calcoaceticus*-*A. baumannii* complex (3, 40). The results of our study not only support the application of ARDRA for genospecies determination of *A. calcoaceticus*-*A. baumannii* complex isolates but also suggest that most of the genospecies could be identified for clinical consideration using only three restriction enzymes. There were still two isolates for which the genospecies could not be determined in this study. According to molecular pattern comparison, one isolate could be genospecies 13BJ or 14TU *A. haemolyticus*/*A. johnsonii*, and the other one could be genospecies 5 (*A. junii*), 15BJ, 16BJ, or 17BJ; these genospecies currently have less clinical importance. In a clinical microbiology laboratory, one could always consider the use of more restriction enzymes in ARDRA for better identification.

Conclusion. This study indicated the current emergence of carbapenem-, fluoroquinolone-, aminoglycoside-, and cephalosporin-resistant *A. calcoaceticus*-*A. baumannii* complex isolates in Taiwan. Moreover, ARDRA was shown to be a useful tool to study the genetic diversity of *A. calcoaceticus*-*A. baumannii* complex isolates for clinical consideration. Clinicians need to aware that not only does the so-called "*A. baumannii*" have clinical importance but other genospecies also may have different antimicrobial resistance and virulence potential characteristics. Beyond these major observations, after further analyzing our results by year, it was shown that *A. baumannii* genospecies isolates resistant to all 12 antibiotics tested were dominant in 2007 and 2008. However, in 2009, isolates were still mainly of the *A. baumannii* genospecies, but most of them were susceptible to all 12 antibiotics tested. The reason for such a change remains unknown and needs further investigation.

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